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Development
Growth & Differentiation

Pluripotent Embryonal Stem Cell Lines Can Be Established from Disaggregated Mouse Morulae

(mouse embryonal stem (ES) cells/disaggregated morulae/in vitro development/teratoma)

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Mouse pluripotent embryonal stem (*ES*) cell lines hitherto have been conventionally isolated from the 'inner cell mass' of mouse blastocysts. In this report, I describe a new and simplified method for establishing pluripotent cell lines from mouse morulae of the 16- to 20-cell stage, which were disaggregated by the use of EDTA. From 17 cell lines established in such a way, 7 were characterized with respect to their differentiation potential:

(i) When injected into syngeneic mice, the cells gave rise to solid, fully differentiated teratomas representing derivatives of all three germ layers. (ii) When cultured in suspension in vitro, the cells were abl to differentiate into complex organized 'embryoid bodies' analogous to mouse early postimplantation embryos. These results strongly imply that embryonal stem cell lines isolated from mouse morulae ar highly homologous to conventionally isolated *ES* cells.

In addition, my results indicate that murine pluripotent embryonal stem (*ES*) cell lines can be derived with more ease and higher efficiency from disaggregated morulae than from the 'inner cell mass' of blastocysts.

Introduction

Pluripotent embryonal stem (*ES*) cells have been established by culturing and propagating the 'embryoblast' of the mouse blastocyst on a fibroblast feeder cell layer (1, 4, 7, 13, 22). In contrast to the most currently available embryonal carcinoma (*EC*) cells, which are the pluripotent stem cells of teratocarcinomas (21), *ES* cells are isolated exclusively in vitro. *ES* cells have been shown to exhibit several principal advantages compared to *EC* cells:

Firstly, *ES* cells keep their diploid karyotype, when cultured at appropriate culture conditions (7). Secondly, *ES* cells cultured in suspension show a high level of organized 'in vitro development', resulting in embryo-like structures (4, 6). Thirdly, *ES* cells with high frequency form germ line chimeras, when reinjected into mouse blastocysts (2, 8). From these points of view, *ES* cells (rather than *EC* cells) resemble the pluripotential cells of the mouse embryo (19), and can therefore be considered as a model system suitable for the analysis of different aspects of murine development.

In this study, I have established a new and simplified method for the high efficient isolation of *ES* cell lines by using EDTA-disaggregated mouse morulae of the 16- to 20-cell stage.

Materials and Methods

Mice, Embryos, and Establishment of disaggregated morulae-derived ES cell lines. Female mice of strains 129 Sv/J or C57Bl/6 were superovulated by subsequent injections of 5 units of pregnant mare's serum gonadotropin (PMSG, Sigma Chemicals) and 5 units of human chorionic gonadotropin (hCG, Sigma Chemicals) within a 48 hour interval. Females detected to have a vaginal plug in the morning after being mated with C57Bl/6 males were designated to be pregnant of day 1. Morulae were collected in the morning of day 3 of pregnancy by flushing the oviduct with 'embryo medium' (Dulbecco's modified Eagle medium (DMEM, Gibco) containing 10% foetal calf serum, 10% newborn calf serum (Gibco), and 10^{-4} M 2-mercaptoethanol). Compacted morulae composed of approximately 16 cells (as roughly assessed by size and morphology of the embryos) were selected for further manipulation. The embryos were rinsed three times in phosphate-buffered saline (PBS). Zonae pellucidae were removed by

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treatment in 0.5% Pronase-solution, as described (16). Subsequent dissociation was performed by gently pipetting the morulae in PBS containing 0.3% EDTA, until single blastomeres were obtained (about 3 min). The procedure had to be carefully monitored microscopically to prevent demage to the embryos. Separated blastomeres were washed 5 times in 'embryo medium' and cell numbers were recorded for each disaggregated embryo. Blastomeres from embryos consisting of 16 to 20 cells were cultured in 'embryo midium' on mitomycin C-treated embryonic fibroblasts (5) at 37°C, in a humidified 10% CO_2-atmosphere. Medium was changed every two days. Cell clones by morphology identified to be stem cell-like were picked and propagated.

ES cell culture and differentiation in vitro. *ES* cells were routinely cultured on mitomycin C-treated embryonic fibroblasts in 'embryo medium'. After removal from the 'feederlayer', *ES* cells were allowed to differentiate in suspension in bacterial dishes as described (4, 6). Standard medium (15% foetal calf serum in DMEM) was changed in two day intervals.

Histology and immunofluorescence. Tumors and 'embryoid bodies' were fixed in Bouin's solution, embedded in paraffin, sectioned, and stained using standard procedures. Indirect immunofluorescence staining with the monoclonal antibodies ECMA-7 (19) and TROMA-1 (3) were performed as described (6). ECMA-7 and TROMA-1 were a kind gift from Dr. R. Kemler (Max Planck Institut für Immunobiologie, Freiburg, FRG).

Results

Establishment of disaggregated morula-derived ES cell lines

Embryonal stem cell lines were established from selected morulae derived from crosses of ((C57Bl/6×129 Sv/J), or $(C57Bl/6)^2$) mice. In three individual experiments 89 morulae were prepared. Of these, 71 morulae had undergone compaction and were dissociated in a 0.3% EDTA-solution, as described in Materials and Methods. 13 embryos contained less than 16 blastomeres. 46 morulae were found to consist of 16 to 20 blastomeres, with an average number of 17 per disaggregated morula. 7 embryos contained more than 20 cells, and 6 morulae which were damaged during the dissociation procedure were discarded (Table 1).

After being extensively washed in medium, the disaggregated blastomeres were transferred into 24-well tissue culture plates coated with mitomycin C-treated primary fibroblasts. In all cases, the cells of one individual morula were seeded together in one well without contact to other blastomeres. Within 3 hr after being transferred to the culture wells, the blastomeres started to attach to the "feeder cells". This process was competed 16 hours later, when the blastomeres could only poorly be recognized in the wells. Within 24 to 48 hours of culture, groups of differentiated cells, mainly showing endoderm-like morphology, were clearly distinguishable in all wells. These cells showed strong staining with a monoclonal antibody directed against intermediate filament-like proteins (TROMA-1, Fig. 1), thus with high probability representing derivatives of either primitive endoderm or trophectoderm. No stem cell-like colonies were detectable at that stage.

After approximately 48 to 72 hr of culture, in 17 cases multiple cell clones (1 to 4 per well, see Table 1) exhibiting stem-cell morphology grew out (Fig. 2A). The cell clones of each well were picked, combined, and propagated. Finally, 17 cell lines could be established as described in (4).

In several control experiments, from 108 blastocysts cultured conventionally according to (4), only 9 *ES* cell lines were isolated, representing a yield of about 8%. In contrast, from a total of 46 dissagregated morulae, 17 *ES* cell lines had been established. This corresponds to an efficiency of

Table 1.

genotype of crosses	No. of experiments	Total No. of morulae	No. of morulae composed of 16 to 20 cells	Average number of blastomeres per morula	Ralationship morulae/ stem cell colonies (1)	(2)	(3)	(4)[c]	No. of stem cell lines isolated
(C57Bl/6)× 129Sv/J)	2	41[a]	21 (51.2%)	17.4	1	3	1	2	10 7[a]
		20[b]	8 (40.0%)	16.2	2	—	1	—	3[b]
$(C57Bl/6)^2$	1	28	17 (60.7%)	18.1	2	2	1	2	7

[a,b]: different experiments
[c]: (1), (2), (3), (4) represents the amount of stem cell colonies found per morula. The numbers stand for those morulae from which a stem cell lines were established.

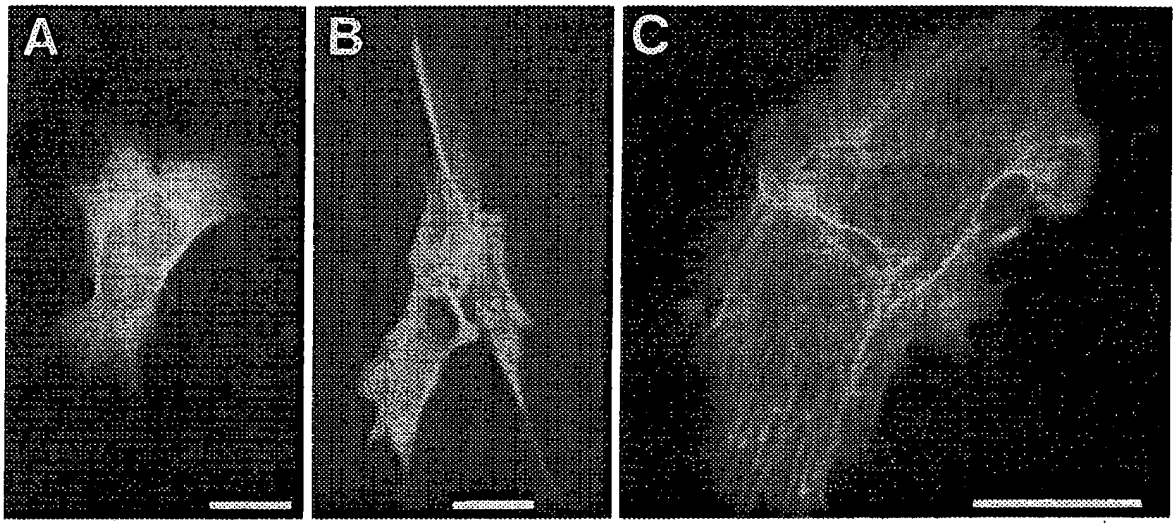

Fig. 1 Groups of differentiated, endodermal cells derived from isolated blastomeres of 3 individual EDTA-dissagregated mouse morulae (A-C), which were cultured on mitomycin-C treated primary embryonic fibroblasts for 48 hr. Immunofluorescence staining with TROMA-1, a monoclonal antibody directed against intermediate filament-like proteins. Bars: 50 μm.

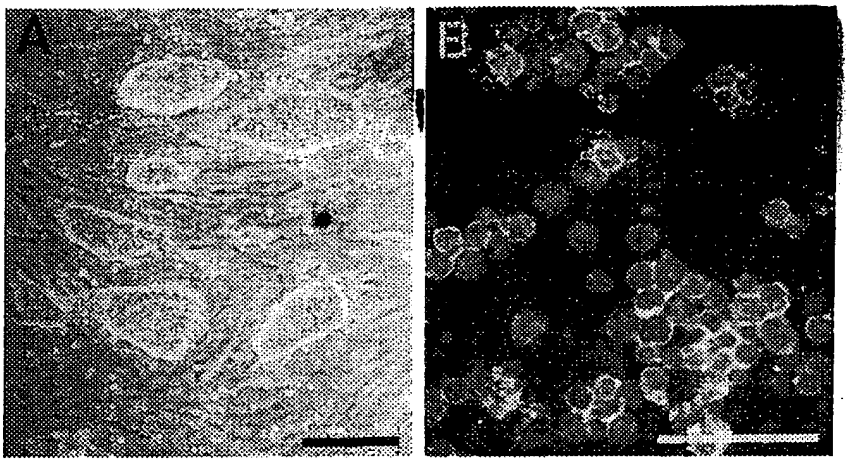

Fig. 2 (A) Colonies of undifferentiated morula-derived embryonal stem cells on a primary embryonic fibroblast 'feeder cell layer' after two passages of amplification. (B) Immunofluorescence staining with ECMA-7, a monoclonal antibody detecting a surface antigen specifically expressed on undifferentiated cells. Bars: 50 μm.

about 37% (cell lines obtained per amount of morulae used).

As long as the morulae-derived cells were maintained on a dense fibroblast 'feeder layer', they expressed an undifferentiated phenotype, as determined by morphology (Fig. 2A) and by immunofluorescence staining with an antibody marker specific for undifferentiated cells (Fig. 2B). Meanwhile, the cells have been cultured for nearly two years, without any significant change in morphology, growth characteristics, karyotype, or differentiation capacity (see below).

Pluripotency of morulae-derived stem cells "in vivo"

In order to characterize these morulae-derived cell lines with respect to their differentiation potential, about 2×10^7 cells were subcutaneously injected into syngeneic mice (either 129 Sv/J or C57Bl/6). This was done for 7 of the 17 cell lines established. In all cases, the cells were found to be highly pluripotent: within 3–5 weeks solid teratomas developed which by subsequent characterization were shown to be composed of derivatives of all three germ layers. Fig. 3 shows some representative histological sections of such teratomas,

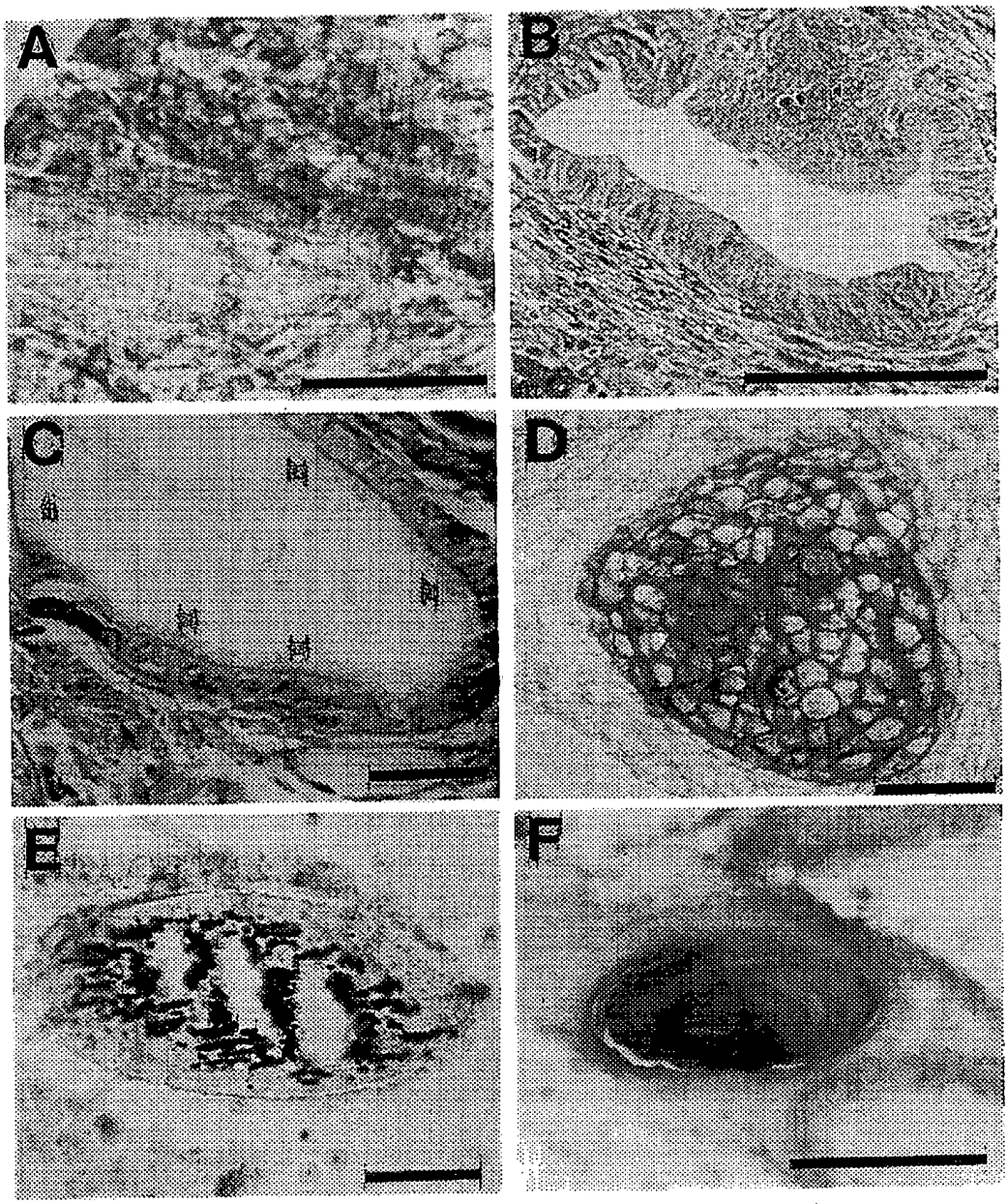

Fig. 3 Histological sections of tumors induced with morula-derived embryonal stem cells. Hematoxylin/eosin staining indicates the presence of cross-striated muscle (A), glandular (B), and ciliated (C, arrows) epithelia. Non-calcified (D) and calcified cartilage (E) were found by means of alcian blue and 'van Kossa' staining, respectively. Figure 3F shows a keratin sworl visualized by using the 'Mallory' staining technique. Bars: 100 μm.

obtained from 3 different morula-derived stem cell lines. A wide variety of cell types and tissues including cross-striated muscle, calcified and non calcified cartilage, epithelia, keratin sworls, and others were seen.

Pluripotency of morulae-derived stem cells in vitro

Five of 7 *ES* cell lines, which had been proven to be highly pluripotent in vivo, were kept in suspension cultures (4, 6) for 3 weeks to analyze their in vitro differentiation capacity. Within 3 days of culture, about 90% of the cells had formed aggre-

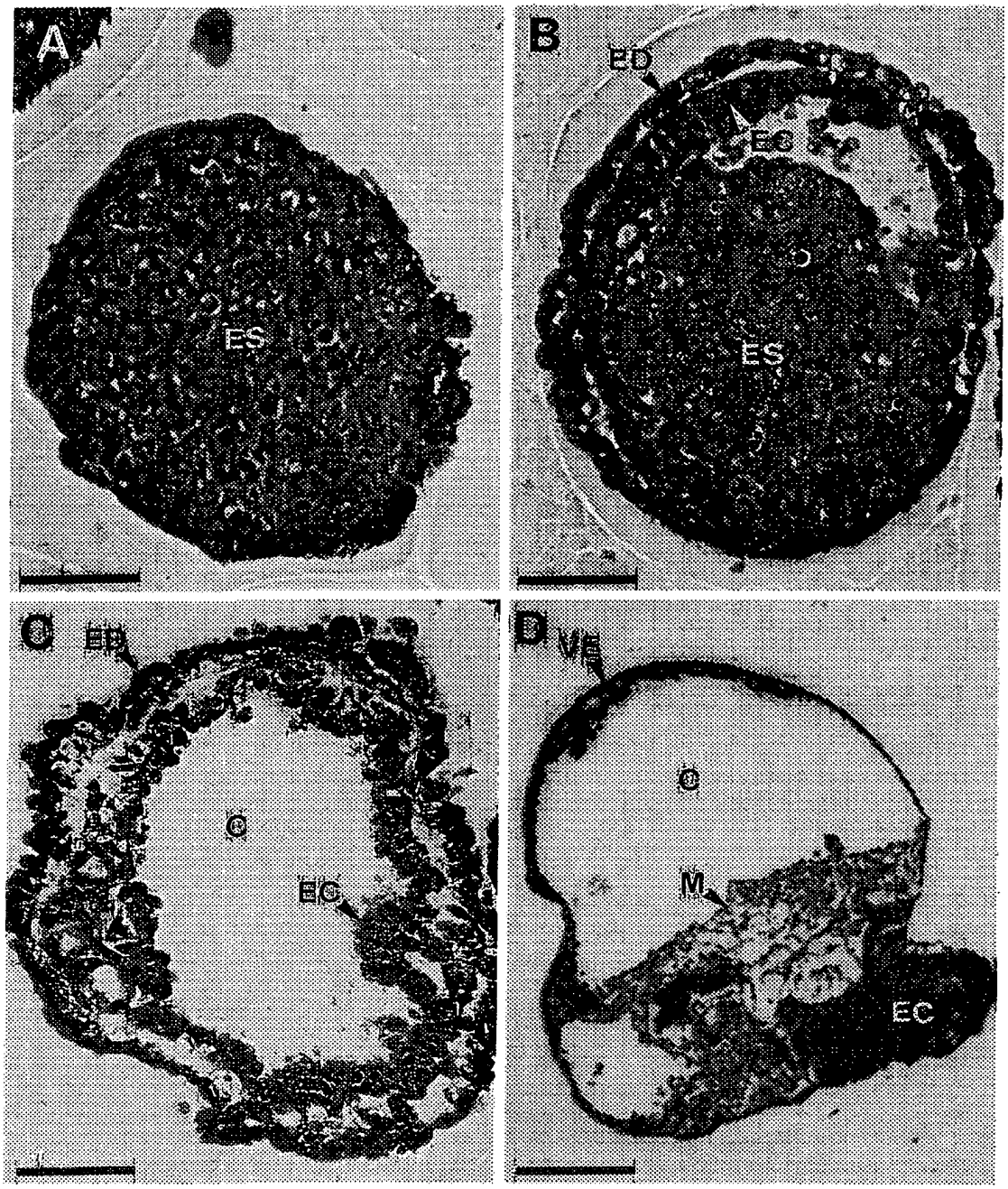

Fig. 4 *In vitro* differentiation in suspension culture. Histological sections of 'embryoid bodies' being cultured for 3 days (A), 6 days (B), 9 days (C), and 15 days (D). Sections were stained by 'Mallory'. ES: Stem cells, ED: endoderm, EC: ectoderm, VE: visceral endoderm, C: cavity, M: mesoderm. Bars: 50 μm (A, B, C), 100 μm (D).

gates (so-called 'embryoid bodies', Fig. 4A). After 6 days in about 80% of these aggregates columnar ectoderm-like cells were bordered by a basal lamina and an outer layer of endoderm (Fig. 4B). By 9 days of culture the endodermal cells shifted to the visceral endoderm type along with the transition from complex to cystic 'embryoid bodies'. This is consistent with the expression of

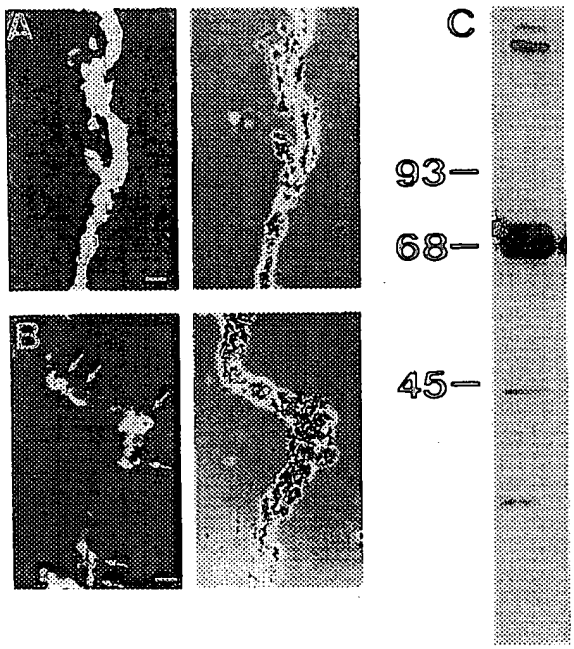

Fig. 5 Immunofluorescence staining of cryostat sections of endodermal parts of '15-days embryoid bodies' generated from morula-derived stem cells. Expression of α-foetoprotein (A) can be seen in nearly all cells. Cells containing transferrin (B) are indicated by arrows. Corresponding phase contrast photographs are shown in the right fields. Bars: 50 μm. (C) Autoradiograph of gelelectrophoretically analyzed ^{35}S-methionine-labeled fluid content of '15-days embryoid bodies'. The presence of α-foetoprotein (black arrow), and transferrin (white arrow), was confirmed by immunoprecipitation (not shown). Molecular weights are given in kiloDalton (kDa).

transferrin and α-fetoprotein in the endodermal cells, as demonstrated by indirect immunofluorescence staining (Figure 5A, B). After 15 days of culture, about 30–50% (depending on the cell line) of cystic structures were present (Fig. 4C, D). Gelelectrophoretical analysis of ^{35}S-methionine-labeled proteins from cystic 'embryoid bodies' fluid content revealed similarities to the fluid content of 12-day embryonic visceral yolk sac (15, 20, 4, Fig. 5C), thus indicating the ability of the morula-derived *ES* cells to develop into structures similar to the postimplantation embryo. In addition, after 6 to 8 days in suspension culture in about 10% of the 'embryoid bodies' rhythmically contracting cells were observed, indicating the presence of muscle-type cells. In parallel, in histological sections loosely attached cells with mesodermal morphology could be seen (M, in Fig. 4D). When the morula-derived *ES* cells were allowed to attach to tissue culture plates without 'f eder layer', they differentiated into a variety of epithelial structures, neural-like cells, trophoblastic giant cells, smooth or cross-striated muscle, and several others more (Fig. 6).

Discussion

Mouse pluripotent embryonal stem (*ES*) cell lines have been extensively used as a model system for the analysis of murine development. *ES* cells were applied in the investigation of parthenogenesis (11), of chromosome abnormalities (17), of targetted gene correction (5), and of developmentally regullated gene expression (6). In addition, *ES* cells have been described as a very powerful tool to generate transgenic mice (9, 18), and have been isolated from embryos carrying recessive lethal mutations (12, 14).

In this report I describe a simple and efficient method for establishing pluripotent embryonic stem cell lines from EDTA-disaggregated mouse morulae. Of 46 embryos, 17 cell lines were succesfully isolated, representing a yield of about 37%. Only 9 *ES* cell lines, in contrast, could be dirived from the 'inner cell mass' of 108 blastocysts (about 8% efficiency) which were treated conventionally. This difference in yields might be due to the fact that the stem cell blastomeres of the morulae are cultured single. Thus, they seem to be less exposed to differentiation stimulating influences, as, for example, the contact to cells which are already committed to the endodermal fate. Interestingly, in most cases, multiple stem cell colonies grew out of one disaggregated morula, obviously representing derivatives of several pluripotent morula stem cells. Thus, 16- to 20-cell stage morulae seem to contain about 4 stem cells, since more than 4 cell clones were never found per morula. This finding might be important to get a hand on pluripotent cell lines derived from single embryonal stem cells (perhaps even of different developmental stages/fates) in future.

All *ES* cell lines isolated and characterized in this study showed characteristics similar to those reported earlier for blastocyst-derived *ES* cells (1, 4, 6, 7, 13, 22). All of the cell lines analyzed had normal diploid karyotypes with 40 chromosomes in more than 70% of the cells (data not shown). By inducing teratomas in vivo, a wide variety of different cell types and tissues could be identified. When cultured in vitro the morula-derived cells were shown to differentiate into cell types representing all three germ layers. Moreover, at appropriate culture conditions, complex and cystic

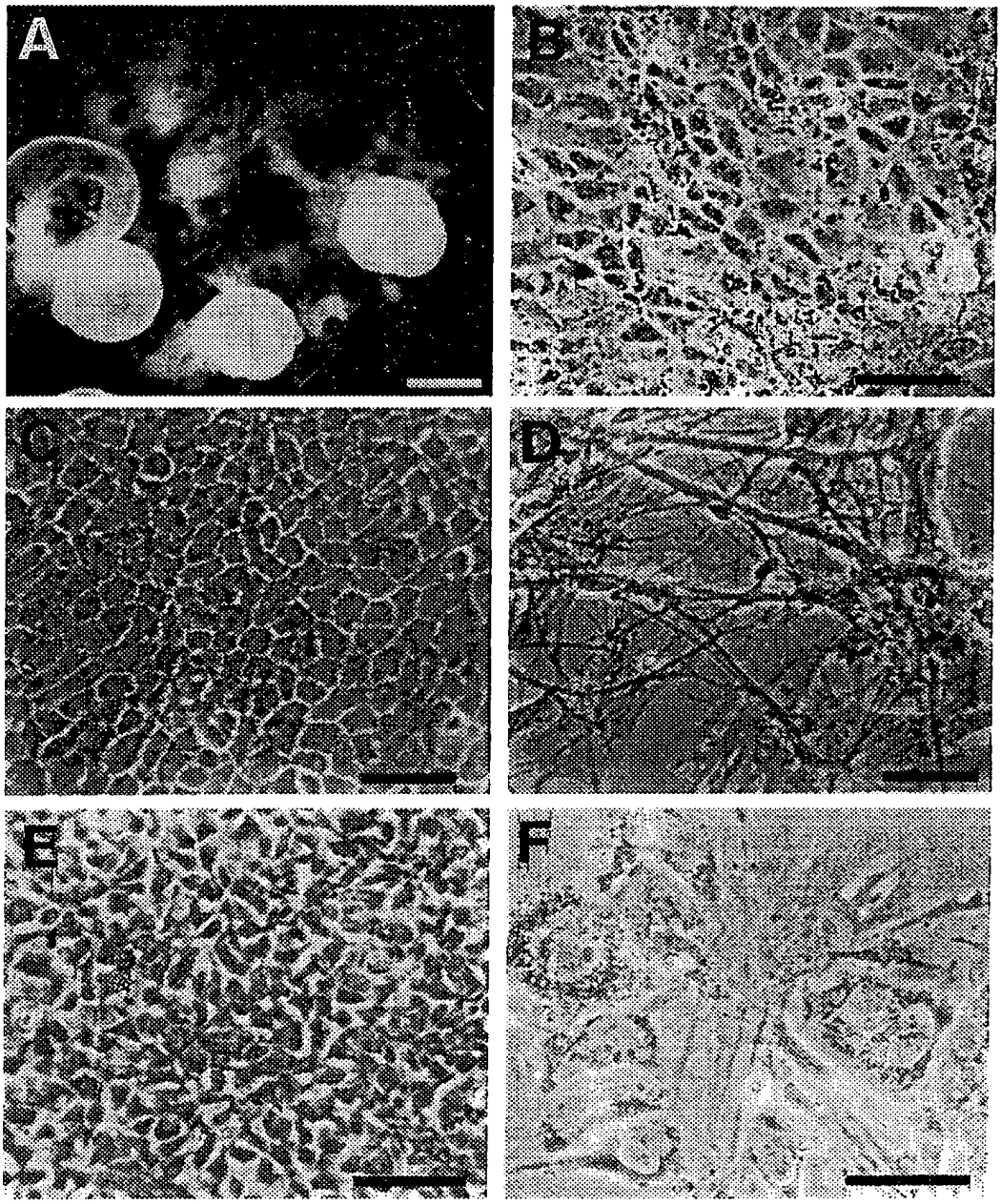

Fig. 6 Differentiation potential of morula-derived stem cells in plastic culture dishes without "feeder layer" cells. (A) Dark field photography of a 19 days differentiated culture showing large cystic structures on a sheet of differentiated cells. Different epitheloid cell types were seen after 4 (B), and 14 (C) days of culture. Nerve cells (D) could be observed after 3 weeks of differentiation. Mesodermal cells (E), and trophoblastic giant cells were found after 9, and 5 days respectively, in culture. Bars: 100 μm (B-F), 1 mm (A).

'embryoid bodies' were found. These structures frequently contained rhythmically beating cells presumably representing myocard (4), and strongly resembled the early postimplantation embryo. These results imply that *ES* cell lines derived from disaggregated morulae are highly pluripotent, as are their relatives established from the 'inner cell mass' of mouse blastocysts. Reinjection of moru-

la-derived pluripotent stem cells into mouse blastocysts and the eventual subsequent generation of transgenic mice should provide a powerful means to prove whether this statement holds.

One of the major advantages of the *ES* cell system lies in the fact that genetically defined cell lines can be established by using single embryos as sources for the isolation procedure. Thus, the generation and analysis of cell lines carrying homozygous mutations (for example) becomes feasible (12, 14). With this respect, and for several other purposes, too, it becomes of particular interest to easily get a hand on a sufficient amount of *ES* cell lines. Since such cell lines can be derived from blastomeres of disaggregated morulae with high efficiency, this new method eventually offers an appropriate tool for the future isolation of normal and/or mutant *ES* cell lines.

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